Reference: *Biol. Bull.*, 149: 568–579. (December, 1975)

DYNAMICS OF JUVENILE HORMONE ACTION IN LARVAE OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)

H. FREDERIK NIJHOUT[1]

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138; and Department of Zoology, University of Washington, Seattle, Washington 98195

In insects the maintenance of larval characters depends on the presence of active *corpora allata* (CA) (Williams, 1961; Wigglesworth, 1970). The implication is that adequate titers of juvenile hormone (JH) in the hemolymph and tissues are necessary for the maintenance of the larval state. The *status quo* action of JH (Williams, 1963a) has, until recently, been the only well established role for this hormone during larval life. We could regard this role of JH as a "passive" one since its action becomes evident only when an ecdysone-induced molt occurs. In apparent contrast stands the positive role of JH in final-instar larvae of certain Lepidoptera where JH inhibits the secretion of the prothoracicotropic hormone (PTTH) by the brain (Nijhout and Williams, 1974b). It is of interest, therefore, to consider in further detail the requirement for JH in these two processes. The approach taken in the present study was the premature elimination of the source of JH in final instar larvae. The expectation was that such action would lead to an early release of PTTH and would also allow ecdysone to induce prematurely the switchover from larval to pupal development (Truman, Riddiford and Safranek, 1974). The surprising finding was that the mere absence of JH was not a sufficient condition for either of the above-mentioned events. An unidentified time-dependent process must apparently be completed to eliminate the effects of JH on the target tissues.

Materials and Methods

Rearing procedure

Larvae of *Manduca sexta* were reared as described by Truman (1972). All experimental animals were kept at 25° C under a short-day (12L: 12D) photoperiod regimen.

JH extraction

Hemolymph. Isolated abdomens were prepared by ligating CO_2-anesthetized larvae between the 2nd and 3rd abdominal segments with waxed dental floss. The part of the larva anterior to the ligature was always removed. Ligations were performed 4–5 hours after lights-on. Hemolymph from unligated larvae was taken at the same time. For JH extraction the hemolymph of 12–16 larvae was pooled and 7 ml of this sample was extracted and purified according to the procedure

[1] Present address: Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20014.

of Fain (1975). The final lipid residue was dissolved in 28 μl of cyclohexane and stored at −20° C. All extracts were assayed within five days. By suitable tests it was possible to show that no measurable activity was lost during this period.

Blood-free carcass. Anesthetized larvae were slit open, the gut and adhering Malpighian tubules were removed, and the carcass was blotted with filter paper. Fat body, muscle and epidermis were removed from 10–14 such carcasses by means of a roller technique devised by Dr. J. S. Bjerke (Harvard University, personal communication). The pooled tissues were disrupted by sonication at 90 W for a total of 60 sec. Seven ml of the brei were extracted according to the procedure of Fain (1975). Forty μl of the lipid residue were partially purified on glass-backed, silica gel, thin layer chromatography plates (EM laboratories). Chromatograms were developed four times in the same direction with 20% ether-hexane. The portion of the chromatogram corresponding to the position of a tritiated C18-JH (New England Nuclear, 11 C per m mole) marker was eluted with distilled ethyl acetate. After evaporation of the ethyl acetate the lipid residue was dissolved in hexane and passed over a column of activity IV alumina (Woelm). The hexane was then evaporated off and the residue dissolved in an equal volume of cyclohexane and stored at −20° C.

JH assay

The JH extract was assayed on larvae of a black strain of *Manduca* isolated from a mutant which arose spontaneously in our stock. This mutant is expressed as a deficiency in the secretion of JH by the larval CA during the molt to the 5th (final) larval instar (Safranek and Riddiford, 1975). Sufficient JH is present at the time of apolysis to ensure a larval molt, but 17–25 hours later, when the JH titer in the blood of wild type larvae declines to a low level it falls to undetectable levels in the mutant (Safranek and Riddiford, 1975) so that no JH is present during the critical period for normal pigmentation (Truman, Riddiford and Safranek, 1973). The black phenotype can be completely reversed by application of JH during this critical period resulting in larvae of green (wild type) coloration (Safranek and Riddiford, 1975). The response of the black mutant to exogenous JH is identical in all respects to that of larvae used in the black larval assay of Truman *et al.* (1973). Further description of the black mutant is given by Safranek and Riddiford (1975). The bioassay and scoring system are described in detail by Fain and Riddiford (1975).

One microliter of the hemolymph extract or 3 μl of the blood-free carcass extract was applied to each of 20–25 test larvae. Their response was scored three days later. The mean score and 95% confidence limits were calculated for the combined data of three replicates of each experiment. Assay of a dilution series of a zero hour sample produced a dose-response curve that paralleled the one for C18-JH. All data were converted to C18-JH equivalents on the basis of a dose-response curve determined by Fain (1975). Although C18-JH has not been detected in *Manduca* (Judy, Schooley, Dunham, Hall, Bergot and Siddall, 1973), the activity of this hormone has been found to be identical to that of C17-JH in the various bioassays developed on *Manduca* (Riddiford and Ajami, 1973; Nijhout and Riddiford, 1974). A proper dose-response curve for C17-JH could not be

prepared due to the unavailability of enough of this hormone. The CA of *Manduca* adult females *in vitro* produce C16-JH and C17-JH in roughly equal proportions (Judy *et al.*, 1973), but C17-JH is 200–300 times more active on *Manduca* than C16-JH (Riddiford and Ajami, 1973; Nijhout and Riddiford, 1974; Fain, 1975). It is therefore likely that C17-JH accounts for virtually all of the JH activity in *Manduca* larvae. The C18-JH equivalence per ml of the original hemolymph sample was calculated assuming an extraction efficiency of 90% (Fain, 1975).

β-ecdysone infusion

Infusions of β-ecdysone were performed on larvae paralyzed with tetrodotoxin (TTX) as described by Nijhout and Williams (1974b). Each larva was infused with 40–45 μg of β-ecdysone (as a 0.5 μg/μl solution in 10% isopropanol) over a period of 20 hours.

Parabiosis

Parabioses were performed on larvae paralyzed with 3 μg/gram body weight of TTX. Where applicable, isolated abdomens were prepared immediately prior to parabiosis. Larvae were joined at the 8th abdominal segment. Prior to the operation a ring of Tackiwax (Cenco) was applied around the area where the incision was to be made. This was necessary because wetting of the cuticle after incision made it difficult to obtain a good seal when the larvae were joined. A hole of about 4 mm in diameter was cut inside the wax ring and a few crystals of a mixture of streptomycin and reduced glutathione were placed in the wound. Two larvae were then lightly pressed together until the wax rings touched and all air bubbles had been expressed. Additional melted Tackiwax was then applied to seal the two larvae together.

Allatectomy

Allatectomies were performed as described by Nijhout and Williams (1974b). All larvae were kept without food subsequent to the operation.

Results

The half-life of endogenous JH in final-instar larvae

Hemolymph JH titers were determined at various times after ligation for abdomens isolated from 5th instar larvae weighing 3.0–3.5 grams. The zero hour sample was taken from unligated larvae. The results are illustrated in Figure 1. One half hour after ligation a marked decline in JH titer had become evident, and by two hours after ligation JH was no longer detectable. The slope of the decay curve indicates that the half-life of hemolymph JH in these isolated abdomens was about 25 minutes. The JH titer in feeding larvae weighing 3.0–3.5 grams was the equivalent of 1.1×10^{-3} μg C18-JH per ml of hemolymph.

Precise quantitative determination of the JH content of blood-free carcasses proved to be impossible because the oily nature of the extract prevented the precise localization required by the scoring system of the black larval assay. Positive

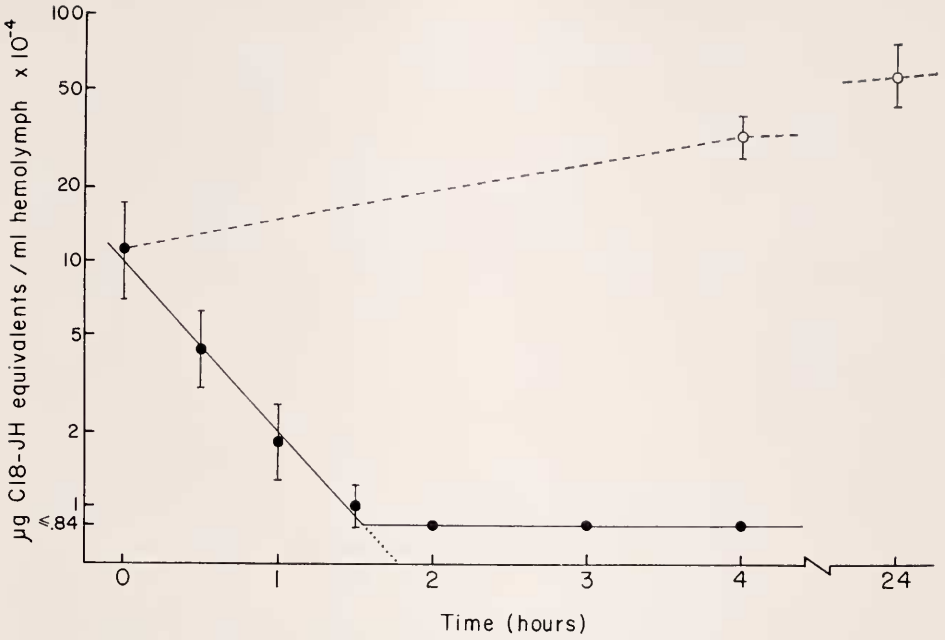

FIGURE 1. Mean JH titers in final (5th) instar larvae weighing 3.0–3.5 grams. Filled circles represent isolated abdomens ligated at 0 hours; open circles, starved larvae. The bars indicate the 95% confidence limits of the mean response of 3 replicates of 20–25 test larvae. Titers below 84 picograms/ml hemolymph cannot be detected by the assay method used. Therefore, the horizontal portion of the graph for isolated abdomens indicates undetectable JH.

scores consisting of a faint greening in the area where the assay material was applied were obtained from 86% (n = 22) and 48% (n = 21) of the assay larvae in each of two replicates with extracts of intact larvae weighing 3.0–3.5 grams. No JH was detectable in blood-free carcasses of isolated abdomens of such larvae by four hours after ligation.

When 3.0–3.5 gram larvae were starved, their hemolymph JH titers rose significantly and remained elevated for at least 24 hours (see dashed line in Figure 1). Such a rise in JH titer was not observed in feeding larvae (Nijhout and Williams, 1974b).

Persistence of JH effects after elimination of its source and in the absence of extractable JH

Nijhout and Williams (1974b) have shown that when final (5th) instar larvae of *Manduca* are induced to molt with exogenous ecdysone, the nature of their response closely reflects the titer of JH in the hemoymph. Thus, in the presence of JH, apolysis takes place initiating a larval molt. By contrast, in the absence of JH the larva is induced to expose its dorsal vessel, a prodromal symptom of pupation. In the present study it was, therefore, surprising to find that when β-ecdysone infusion of small isolated abdomens was initiated 24 hours after

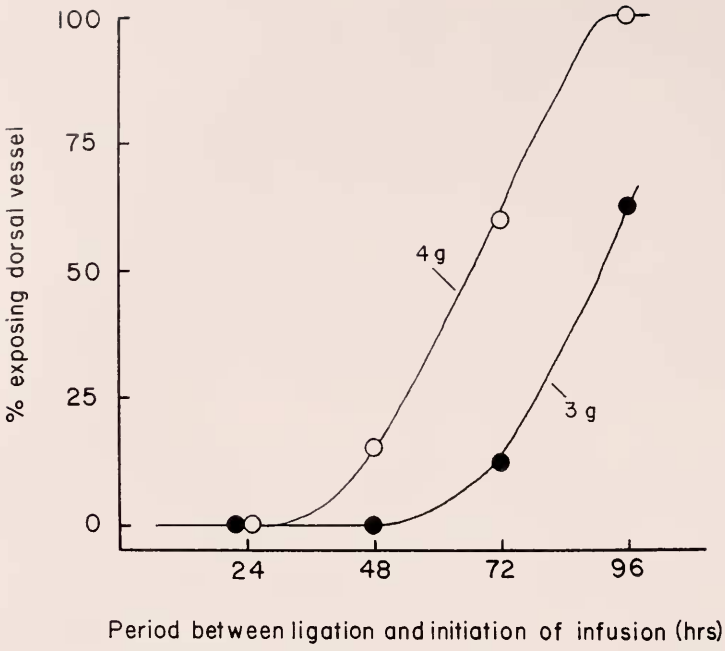

FIGURE 2. Response of isolated abdomens of 3 and 4 gram final instar larvae to infusion with β-ecdysone at various times after ligation. Each point represents 5–11 abdomens. All abdomens which did not show exposure of the dorsal vessel underwent a larval molt.

ligation, they underwent apolysis and proceeded to deposit a normal larval cuticle. Clearly, this larval response occurred long after the disappearance of JH from the hemolymph.

This phenomenon was further investigated on 5th instar larvae weighing 3.0 and 4.0 grams. Abdomens of these larvae were isolated by ligation at time zero and then stored at 25° C for 24, 48, 72 or 96 hours prior to the initiation of the β-ecdysone infusion. The results, illustrated in Figure 2, show that JH effects persisted for almost 3 days in 50% of the abdomens from 4.0 gram larvae and somewhat more than 3.5 days in 50% of the abdomens isolated from 3.0 gram larvae. It should be noted here that those abdomens that initiated a larval molt upon infusion after 72 and 96 hours did not deposit a completely normal larval cuticle. Rather, the new cuticle was smooth, yellowish, and devoid of the setae which are normally present on larval cuticle. Moreover, the spiracles of these individuals were distinctly pupal.

In 5th instar larvae of *Manduca* the presence of JH is known to inhibit the release of the prothoracicotropic hormone (PTTH). The release of PTTH that initates pupation occurs during the first photoperiodic gate after JH has disappeared from the hemolymph (Nijhout and Williams, 1974b). However, when 3.5–4.0 gram larvae were allatectomized, PTTH release occurred about a day later than would be predicted on the basis of the known period required for the clearance of JH from the hemolymph after the larva attains a weight of 5 grams (Nijhout

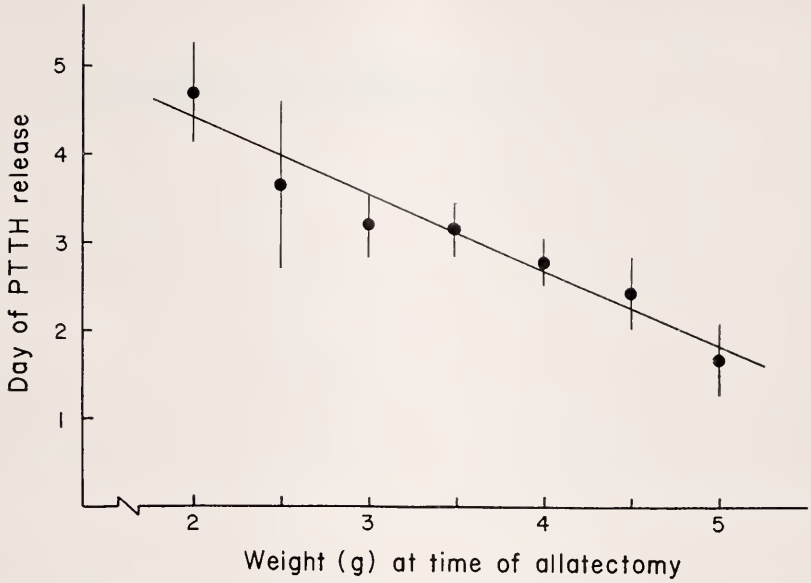

FIGURE 3. The timing of PTTH release in final instar larvae which were allatectomized at various weights and subsequently starved. The day of allatectomy is designated as day 0. The bars indicate the 95% confidence limits of the mean. Each point represents approximately 15 larvae.

and Williams, 1974b). I have investigated this discrepancy in further detail by allatectomizing larvae at various weights, isolating the resulting individuals without food, and noting the day on which PTTH release occurred. Figure 3 summarizes these experiments and shows that the release of PTTH occurred progressively later as larvae were allatectomized at lower weights. PTTH release occurred approximately 2.75 and 3.5 days after allatectomy of 4.0 and 3.0 gram larvae, respectively. These times correspond fairly well with the period required for the disappearance of JH effects in 50% of the isolated abdomens of larvae of similar weights (Figure 2).

All allatectomized larvae exposed their dorsal vessel and proceeded to deposit a perfect pupal cuticle. Forty seven percent ($n = 58$) of the starved, sham-operated control larvae weighing 3.0–4.0 grams molted to larval-pupal intermediates. PTTH release in these control larvae occurred predominantly on days 5–8 after the operation. Few sham operated larvae weighing less than three grams survived to molt, death occurring 7–12 days after the operation.

Parabiotic preparations

Intact small 5th instar larvae, weighing 3.0–3.5 grams were parabiosed to large 5th instar larvae weighing 6.8–8.0 grams. Induced by the secretion of PTTH and ecdysone of the large larva, the small partner invariably molted into a nearly perfect additional larval instar except for the absence of crochets on the prolegs and the presence of small patches of pupal cuticle on the clypeus and

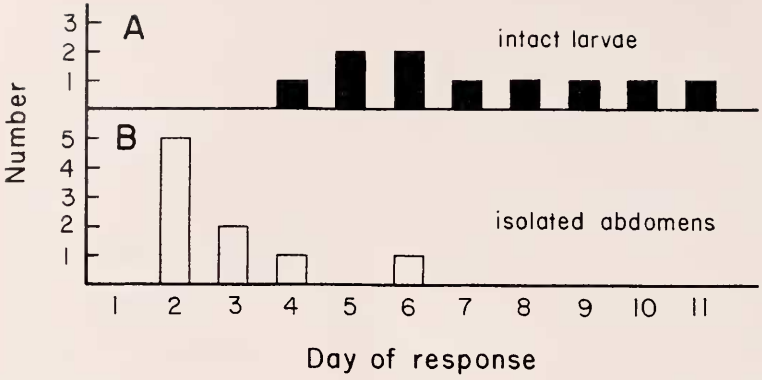

FIGURE 4. Response of final instar larvae weighing 3.0–3.5 grams to ecdysone supplied by parabiosis to 6.8–8.0 gram hormone donors. *A* shows the timing of the response of intact larvae and *B* that of isolated abdomens. *White* represents exposure of the dorsal vessel followed by pupation; *black*, apolysis and deposition of a larval cuticle. Parabiosis was done on day 0. Ligations were performed immediately prior to parabiosis.

anterior to the larval ocelli. The exposure of the dorsal vessel in the large partner and the concomitant onset of apolysis in the small partner occurred on widely scattered days (Figure 4A). This scatter was apparently due to the fact that the JH from the small larva inhibited the release of PTTH in its large partner (Nijhout and Williams, 1974b). In fact, five of ten large donors parabiosed to intact larvae showed retention of some larval characters when they pupated.

By contrast, when isolated abdomens prepared from larvae weighing 3.0–3.5 grams were parabiosed to large intact larvae, the former always exposed their dorsal vessel and proceeded to molt to perfect pupal abdomens. The exposure of the dorsal vessel occurred predominantly on day 2 after parabiosis (Figure 4B). Therefore, the abdomens had been exposed to a pulse of ecdysone on day 1, about 24 hours after the ligation. Evidently the effects of JH had been eliminated in these isolated abdomens during that 24 hour period.

Discussion

The half-life of the endogenous JH activity in isolated abdomens of early 5th instar larvae is about 25 minutes. This exceedingly short half-life is probably not significantly different from the 20–30 minute half-life of exogenous (injected) JH in 5th instar *Manduca* (Ajami, 1973). This was an unexpected finding as it has heretofore been assumed that endogenous JH is somehow protected from rapid degradation, possibly by combination with one or more carrier proteins (Slade and Zibitt, 1972; Sanburg, Kramer, Kezdy, Law and Oberlander, 1975b). Protection of the endogenous JH is nevertheless evident in the abdomens of male *Hyalophora cecropia* (Williams, 1963b; Metzler, Meyer, Dahm and Roeller, 1972; Ajami and Riddiford, 1973) and possibly occurs in the earlier larval instars of *Manduca* where its half-life is longer than in the 5th instar (Fain, 1975). The half-life of JH is likely to be still shorter at a later time in the 5th instar when the titer of JH-esterase increases dramatically

(Weirich, Wren and Siddall, 1973; Sanburg, Kramer, Kezdy and Law, 1975a). Indeed, Slade and Zibitt (1972) report that JH is 85% metabolized after a 5 minute exposure to prepupal blood of *Manduca in vitro*.

Sanburg *et al.* (1975a) have recently demonstrated the existence of two classes of esterases that hydrolyse JH. General esterases are always present in the hemolymph of larvae but are capable of hydrolysing free JH only. On the 4th day of the 5th larval instar there is a sudden appearance of JH-specific esterases in the hemolymph. The latter are able to hydrolyse free JH as well as JH complexed to its binding protein. The appearance of the latter class of esterases coincides with the period of JH decline in 5th instar larvae (Nijhout and Williams, 1974b). It is of interest to note that *in vivo,* in the absence of the JH-specific esterases of Sanburg *et al.* (1975a), the half-life endogenous JH is about 25 minutes and all JH can be cleared from the hemolymph in less than two hours after its secretion ceases (Figure 1). Thus, the JH hydrolysing activity in the hemolymph of a 3 gram larva (on the 2nd day of the 5th instar) is more than sufficient to cause the elimination of JH within the 24-hour period in which it normally occurs (Nijhout and Williams, 1974b). The appearance of the JH-specific esterases on day 4 of the 5th instar does not appear to be a necessity for successful clearance of JH from the hemolymph. An alternate role for the JH-specific esterases is suggested below.

It should be understood that only a marginal amount of JH was detected in the extract from blood-free carcasses of intact early 5th instar larvae. The large amount of lipid coextracted with the JH interfered severely with the assay, even after partial purification by thin layer chromatography. It is, therefore, not rigorously excluded that the inability to detect JH in blood-free carcasses 4 hours after ligation is an artifact of the extraction and assay procedure. It is nevertheless clear that less JH is present in ligated than in intact larvae.

As shown in Figure 1, the titer of JH persisted at high levels when small 5th instar larvae were starved. Indeed, a significant increase in titer was observed, indicating that nutrition may be involved in the maintenance of proper JH titers in these larvae. When 5th instar larvae are starved at a weight of 3 grams, they eventually molt into pupae retaining many larval characters (Nijhout and Williams, 1974a; Nijhout, 1975). These results indicate that in such starved larvae the titer of JH remained elevated for at least 7–8 days.

In view of the short half-life of JH it was surprising to find that isolated abdomens underwent a perfect larval molt when infused with β-ecdysone long after JH had become undetectable in the hemolymph. This persistent effect of JH was not an artifact of the β-ecdysone infusion because an identical infusion induced normal initiation of pupation (exposure of the dorsal vessel) when given a few days after ligation (Figure 2). Furthermore, such infusions never induced larval molting when performed on intact larvae weighing 7.5 grams or more or on isolated abdomens of such larvae; that is, after JH had naturally disappeared (Nijhout and Williams, 1974b; H. F. Nijhout, unpublished results). The close correspondence between the titer of JH in the hemolymph and the response to β-ecdysone infusion demonstrated by Nijhout and Williams (1974b) indicates that when the titer of JH falls below a level detectable by the black larval assay it is also physiologically undetectable by the larva. It is, therefore, difficult to

believe that the persistent effect of JH described in the present paper was due to the presence of molecular JH in the hemolymph at suprathreshold concentrations undetectable by the bioassay. Rather, it seems possible that the developmental program of the larval tissues does not require the continuous presence of JH to maintain its *status quo*. This hypothesis also finds support in the recent work of Fain and Riddiford (Harvard University, personal communication) who have shown a similar persistence of JH effects in isolated abdomens of 4th instar larvae of *Manduca* and, more importantly, in pieces of crochet epidermis cultured *in vitro* in the absence of JH (Fain and Riddiford, 1973; Fain, 1975).

The data in Figure 3 show that the ability of JH to inhibit the release of PTTH (Nijhout and Williams, 1974b) is likewise independent of the presence of molecular JH in the hemolymph. The inhibition of PTTH release was brought about by some persistent effect of JH which could remain active for 4 to 5 days in small (*e.g.* 2 grams) allatectomized larvae (Figure 3). The close agreement between the 50% points of the curves in Figure 2 and the data for the corresponding weight classes in Figure 3 show that the release of PTTH occurred precisely at the time that the persistent morphogenetic effects of JH disappeared. The effect of starvation on the release of PTTH, therefore, appears to be insignificant.

This persistent effect of JH is rather analogous to the situation found by Ohtaki, Milkman and Williams (1968) for the action of ecdysone. We are likely dealing with a fundamental principle of hormone action—namely, the fact that an overt response to a hormone is not possible until a number of biochemical events have taken place in the target tissue. The induction and completion of these unknown events have been termed the "covert effects" of the hormone (Ohtaki *et al.*, 1968) —a term which I shall adopt in this case. These covert effects presumably have a longer half-life than the hormone itself. The data in Figure 2 provide a rough estimate of this half-life as 14–20 hours.

The switchover from a larval to a pupal response upon infusion with β-ecdysone occurs at precisely the time that JH disappears from the hemolymph of normally feeding and growing 5th instar larvae (Nijhout and Williams, 1974b). In view of the short half-life of JH, it is likely that the slow decline in JH titer over the 24 hour period following the attainment of the critical weight of 5 grams (Nijhout and Williams, 1974a, b) closely reflects the secretory activity of the CA. Consequently, it appears that the CA are not instantaneously shut off when the critical weight is achieved. Rather, they begin a decline in activity at that time and become completely inactive only about 24 hours later.

The persistence of the morphogenetic effects of JH in the absence of detectable molecular JH in early 5th instar larvae (Figure 2) stands in sharp contrast to the situation later in the instar when there is a close correspondence between the actual titer of JH in the hemolymph and the response to infused β-ecdysone (Nijhout and Williams, 1974b). A mechanism must therefore exist for the rapid elimination of the covert effects of JH towards the end of the final larval instar when the CA have ceased to secrete JH. Evidence for such a mechanism was provided by the observation that late final instar larvae parabiotically joined to abdomens isolated from smaller (3.0–3.5 g) larvae were able to induce exposure of the dorsal vessel and a normal pupal molt in the latter, at a time when a simple infusion of β-ecdysone would have induced a larval molt. These results suggest that the

large hormone donor supplied more than just ecdysone. It was somehow able to effect a premature elimination of the covert effects of JH in the isolated abdomens. Presumably this occurred by means of a blood-borne factor since the two partners shared a common hemolymph pool. Although there is no appreciable difference in the JH titer of larvae weighing from 2.5 to 4.5 grams (Nijhout and Williams, 1974b), Figure 3 shows the covert effects of JH are eliminated more rapidly in the latter than in the former. These results indicate that the activity of the hypothetical factor increases slowly in the course of the instar.

The possibility that the low level of ecdysone that induces the onset of the wandering stage is also responsible for the swift elimination of the covert effects of JH has been considered (Nijhout, in preparation), and it appears that ecdysone is not directly involved in this process. An attractive alternate hypothesis that could explain the experimental results discussed above presents itself on the hand of the recent publications by Sanburg *et al.* (1975a, b). It is possible that the covert effects of JH are due, not to the accumulation of biochemical effects of hormone activity as suggested above, but rather, to the persistence of tightly bound JH, undetectable by the extraction and assay procedure used. If this is the case, the blood-borne factor produced by the large parabiotic hormone donors could be the JH-specific esterases which are present in the hemolymph of these larvae. This implies that "active" JH is normally sequestered by the target tissues and the function of the JH-specific esterases (Sanburg *et al.*, 1975a, b) could be the elimination of the tightly bound JH in the target tissues rather than the hemolymph JH, the latter being efficiently eliminated even in the absence of JH-specific esterases (Figure 1).

I wish to thank Professors C. M. Williams, J. W. Truman and L. M. Riddiford for helpful discussions and for critical reading of the manuscript, and Dr. A. M. Ajami for advice. I am also grateful to Mrs. M. J. Fain for allowing me access to an early draft of her thesis. This work was supported by the Rockefeller Foundation and Grant GB-26593 of the National Science Foundation to Professor C. M. Williams, and (in part) by a National Institutes of Health Fellowship (No. 1-F22-AM01515-01) from the Institute of Arthritis, Metabolism and Digestive Diseases.

Summary

1. The half-life of endogenous juvenile hormone (JH) in the hemolymph of early 5th (final) instar larvae of *Manduca sexta* is approximately 25 minutes. Two hours after ligation JH is no longer detectable in isolated abdomens.

2. The morphogenetic action of JH as well as its inhibitory action on the secretion of the prothoracicotropic hormone persist for several days after molecular JH has become undetectable in the hemolymph and in blood-free carcasses.

3. Evidence is presented which suggests that the action of JH is mediated by long-lived covert effects and that these covert effects of JH are swiftly eliminated toward the end of larval life, apparently through the action of an unidentified blood-borne factor.

4. Two contrasting hypotheses can explain the persistent covert effect of JH. One suggests that the covert effects are stable biochemical changes in the target tissues induced as a normal part of the molecular action of JH. Alternatively, it is possible that molecular JH persists in an unextractable form, tightly bound to the target tissues. In the latter case, the blood-borne factor that causes the elimination of the covert effects could simply be a JH-esterase.

LITERATURE CITED

Ajami, A. M., 1973. Biochemical studies on the metabolism and biosynthesis of the Cecropia juvenile hormone. *Ph.D. thesis, Harvard University,* 72 pp.

Ajami, A. M., and L. M. Riddiford, 1973. Comparative metabolism of the Cecropia juvenile hormone. *J. Insect Physiol.,* **19**: 635–645.

Fain, M. J., 1975. Studies on larval molting in the tobacco hornworm. *Ph.D. thesis, Harvard University,* 134 pp.

Fain, M. J., and L. M. Riddiford, 1973. *In vivo* and *in vitro* response of larval crochet epidermis to ecdysone and juvenile hormone. *Amer. Zool.,* **13**: 1272.

Fain, M. J., and L. M. Riddiford, 1975. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull.,* **149**: 506–521.

Judy, K. J., D. A. Schooley, L. L. Dunham, M. S. Hall, B. J. Bergot and J. B. Siddall, 1973. Isolation, structure, and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. *Proc. Nat. Acad. Sci. U.S.A.,* **70**: 1509–1513.

Metzler, M., D. Meyer, K. H. Dahm, and H. Roeller, 1972. Biosynthesis of juvenile hormone from 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoic acid in the adult Cecropia moth. *Z. Naturforsch.,* **27**: 321–322.

Nijhout, H. F., 1975. A threshold size for metamorphosis in the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull.,* **149**: 214–225.

Nijhout, H. F., and C. M. Williams, 1974a. Control of moulting and metamorphosis in the the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *J. Exp. Biol.,* **61**: 481–491.

Nijhout, H. F., and C. M. Williams, 1974b. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol.,* **61**: 493–501.

Nijhout, M. M., and L. M. Riddiford, 1974. The control of egg maturation by juvenile hormone in the tobacco hornworm moth, *Manduca sexta*. *Biol. Bull.,* **146**: 377–382.

Ohtaki, T., R. D. Milkman, and C. M. Williams, 1968. Dynamics of ecdysone secretion and action in the fleshfly, *Sarcophaga peregrina*. *Biol. Bull.,* **135**: 322–334.

Riddiford, L. M., and A. M. Ajami, 1973. Juvenile hormone: its assay and effects on pupae of *Manduca sexta*. *J. Insect Physiol.,* **19**: 749–762.

Safranek, L., and L. M. Riddiford, 1975. The biology of the black larval mutant of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.,* in press.

Sanburg, L. L., K. J. Kramer, F. J. Kezdy, and J. H. Law, 1975a. Juvenile hormone-specific esterases in the haemolymph of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.,* **21**: 873–887.

Sanburg, L. L., K. J. Kramer, F. J. Kezdy, J. H. Law, and H. Oberlander, 1975b. Role of juvenile hormone esterases and carrier proteins in insect development. *Nature,* **253**: 266–267.

Slade, M., and C. H. Zibitt, 1972. Metabolism of Cecropia juvenile hormone in insects and mammals. Pages 155–176 in J. J. Menn and M. Beroza, Eds., *Insect juvenile hormones chemistry and action*. Academic Press, New York.

Truman, J. W., 1972. Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol.,* **57**: 805–820.

TRUMAN, J. W., L. M. RIDDIFORD, AND L. SAFRANEK, 1973. Hormonal control of cuticle coloration in the tobacco hornworm. *Manduca sexta:* basis of an ultrasensitive assay for juvenile hormone. *J. Insect Physiol.,* 19: 195–203.

TRUMAN, J. W., L. M. RIDDIFORD, AND L. SAFRANEK, 1974. Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta. Develop. Biol.,* 39: 247–262.

WEIRICH, G., J. WREN, AND J. B. SIDDALL, 1973. Developmental changes of the juvenile hormone esterase activity in the haemolymph of the tobacco hornworm, *Manduca sexta. Insect Biochem.,* 3: 397–407.

WIGGLESWORTH, V. B., 1970. *Insect hormones.* W. H. Freeman and Co., San Francisco, 159 pp.

WILLIAMS, C. M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. *Biol. Bull.,* 121: 572–585.

WILLIAMS, C. M., 1963a. Differentiation and morphogenesis in insects. Pages 243–260 in J. M. Allen, Ed., *The nature of biological diversity.* McGraw-Hill Co., New York.

WILLIAMS, C. M., 1963b. The juvenile hormone. III. Its accumulation and storage in the abdomens of certain male moths. *Biol. Bull.,* 124: 355–367.